



# Application of a master equation for quantitative mRNA analysis using qRT-PCR

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## ABSTRACT

The qRT-PCR has been widely accepted as the assay of choice for mRNA quantification. For conventional practice, housekeeping genes have been applied as internal reference for data normalization and analysis since the technology appeared. However, housekeeping genes vary under different conditions and environmental stimuli and no commonly accepted housekeeping gene references are available. Accurate data acquisition and data reproducibility remain challenging and it is difficult to compare results from different experimental sources. Using yeast and a *Fusarium* fungus as examples, we demonstrate the independent performance of a sole reference gene, *CAB*, designated as a constant manual threshold for data acquisition, normalization, and analysis for multiple plate reactions. A robust master equation based on the *CAB* reference and the set of calibration control genes thereafter was established to estimate mRNA abundance for the same RNA background reactions. A valid range of amplification efficiency between 95% and 100% was observed for the control genes in different RNA background applied on an ABI real time PCR 7500 system. This newly developed robust quality control system provides a reliable means for absolute quantification of mRNA using the qRT-PCR, simplifies the conventional qRT-PCR procedures, and increases data reliability, reproducibility, and throughput of the assay.

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## 1. Introduction

Real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) has been recognized to be the assay of choice among available techniques for mRNA quantification. Gene expression as measured by mRNA dynamics varies in response to different conditions and environmental stimuli. For conventional practice, housekeeping genes have been applied as an internal reference for data normalization and analysis since the technology appeared (Collins et al., 1998; PE Applied Biosystems, 1997). When housekeeping genes are used, a careful measurement must be taken in the selection and use of the reference gene and the interpretation of data under different conditions. With increased concerns of variability of housekeeping genes in response to varied conditions, there is no commonly accepted housekeeping gene reference available (Baker et al., 2005; Barber et al., 2005; ERCC, 2005; Goldsworthy et al., 1993; Mohsenzadeh et al., 1998; Tricarico et al., 2002). Acquisition and processing of qRT-PCR data is dependant upon discretion of individual researchers and thus obtained data from different experiments and sources are often not reproducible. Therefore, it is difficult to compare and utilize results from

different experiments, conditions, or among different studies and different laboratories. Accurate data acquisition and data reproducibility remain challenging. In addition, many micro-plate wells are required for reactions of the control genes in order to construct valid standard curves for quantitative analysis of mRNA for multiple treatments or conditions, which limit reaction space for target gene assays. It is especially true when absolute mRNA quantification is required.

Recently, we developed the first universal external RNA controls for quantitative gene expression analysis that can be applied to both microarray and qRT-PCR platforms (Liu and Slininger, 2007). Such universal RNA controls provide independent and reliable reference and allow comparison of expression data generated from different platforms of microarray and qRT-PCR. Applications of the universal controls on the microarray platform have been well covered. However, the extensive uses of the controls on the qRT-PCR platform and related technical issues have not been fully explored. For example, the application of the universal RNA control is not readily accessible using the built-in software with ABI 7500 system. Variations of amplification efficiency and quantitative gene expression can be observed among different assay runs when inconsistent threshold was applied (Liu, unpublished data). A robust reference and a standard data acquisition procedure for the qRT-PCR platform are needed for multiple plate reactions and multiple users. The objective of this study was to develop a robust standard system derived from the universal RNA control that can serve as a constant

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reference for simplified and efficient absolute mRNA quantification using the qRT-PCR. PCR amplification efficiency was examined fully for this application development. Using yeast and a toxin producing *Fusarium* fungus as examples, we demonstrate the robust performance of the control system compared with selective target gene responses under toxin treated and varied culture timing conditions.

## 2. Materials and methods

### 2.1. Standard of mRNA control

Five external mRNA controls, *ACTB*, *B2M*, *CAB*, *MSG*, and *RBS1*, were synthesized *in vitro* as described previously (Liu and Slininger, 2007). A control mix was prepared as previously described, consisting of accurately calibrated mRNA transcripts of *MSG*, *CAB*, *RBS1*, and *ACTB* at 100 fg, 1 pg, 10 pg, and 1 ng/ $\mu$ l, respectively. This control mix served as a calibration standard. A standard curve was constructed for each set of qRT-PCR run. Reactions with a pair of primers for *B2M* and a non *B2M* template were used to serve as negative controls. Based on a consistent performance, the control gene *CAB* at an mRNA input of 1 pg was designated as a sole exogenous transcript reference to set-up a manual threshold for data acquisition and analysis for each qRT-PCR run. A standard mRNA calibration mix and a laboratory benchtop protocol are available to qualified users by request.

### 2.2. Strains, medium, and cultural conditions

*Saccharomyces cerevisiae* NRRL Y-12632 and *Fusarium sporotrichioides* NRRL 3299 (ARS Cultural Collection, Peoria, IL USA) were used. Cell cultures of *S. cerevisiae* Y-12632 were established on a YM medium (yeast extract 3 g, malt extract 3 g, peptone 5 g, glucose 10 g/l of water) with a 6-h incubation on a shaker at 250 rpm at 30 °C. This time point was considered as 0 h at which cultures were treated with 5-hydroxymethylfurfural (HMF). The culture was added with HMF suspended in a fresh medium at a final concentration of 30 mM and continued incubated for 3 h. Detailed culture and treatment procedures were as previously described (Liu et al., 2005, 2008). A treatment of the same amount of culture and medium without HMF served as a control. *F. sporotrichioides* strain 3299 was maintained on V-8 juice agar and cultured for 8–10 days to establish a culture (Stevens, 1974). Spores were dislodged from the agar plates, counted, and inoculated into 50 ml (250 ml Erlenmeyer flask) of 5GYEP (5% glucose, 0.1% yeast extract, 0.1% peptone) at a concentration of  $1 \times 10^5$  spores/ml. Liquid cultures were maintained at 28 °C, 200 rpm, in the dark. For each species, separated cell cultures were carried out with two biological replications. Duplicated cell samples were taken from each of the replicated cultures for each species.

### 2.3. Cell collection and total RNA isolation

For yeast strain Y-12632, cells were harvested at 0 h and 3 h; and total RNA was isolated for each treatment using a procedure as previously described (Liu and Slininger, 2006, 2007). For *Fusarium* strain 3299, cultures were harvested for RNA isolation at 16 h and 24 h post inoculation. Mycelia were harvested using a Buchner funnel and Whatman#1 filter paper, transferred to liquid nitrogen, and ground to a fine powder. RNA was isolated by the Trizol method (Invitrogen, Carlsbad, CA) following the manufacturer's protocol with a modification of adding 0.1 volume of high salts (1.2 M NaCl, 0.8 M NaCitrate) followed by an equal volume of isopropanol to remove excess polysaccharides. RNA cleanup was performed using a Qiagen RNeasy mini kit (Qiagen Sciences, MD). Ten  $\mu$ g of total RNA was treated with DNase (Turbo-Free, Ambion) following the manufacturer's protocol with the exception that an additional 1  $\mu$ l

of enzyme was added after 20 min of incubation at 37 °C for an additional 20 min. Total RNA was obtained from each of two biological and two technical replications. RNA integrity was verified with a NanoDrop Spectrophotometer ND-100 (NanoDrop Technologies, Inc., Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE).

### 2.4. Test gene selection and primer design

Eight target genes of *S. cerevisiae*, *ADH7*, *CHA1*, *GPM1*, *PDR3*, *PDR5*, *RPN5*, *SNQ2*, and an uncharacterized gene Y63, were selected for test in this study based on previous knowledge (Liu, 2006; Liu and Slininger, 2005; Liu et al., 2008). Except for *GPM1* to be repressed, all other genes exhibit enhanced expression to the HMF treatment. Eight target genes of *F. sporotrichioides* were selected, among which, *TEF1* and  $\beta$ -tubulin, two commonly used as housekeeping genes, were included. Translation elongation factor 1 $\alpha$  was used as a constitutive control in a Southern analysis of gene expression in *F. sporotrichioides* (Peplow et al., 2003) and  $\beta$ -tubulin was recently identified as a reference gene (Alexander et al., 2004). Six other genes, *TRI1*, *TRI4*, *TRI5*, *TRI13*, *TRI15*, and *TRI101*, all involved with the production of trichothecene mycotoxins in *Fusarium* (Brown et al., 2001; Desjardins, 2006; McCormick et al., 1999; Peplow et al., 2003) are considered to be the products of secondary metabolism. The trichothecene genes show limited expression, by Northern analysis, at 16 h post inoculation but are expressed by 23 h (Alexander et al., 2004; Peplow et al., 2003; Proctor et al., 1995). Comparison of the expression of the genes at 16 h and 24 h post inoculation is anticipated to detect a quantifiable increase in expression of the trichothecene genes. Gene-specific primers for the selected genes of each species were designed using Primer3 with manual editing for qRT-PCR assays using SYBR Green (Table 1).

### 2.5. Reverse transcription and real time qRT-PCR

Reverse transcription reaction was prepared by adding 1  $\mu$ l of a Ctrl Mix consisting of a set of the above mentioned accurately calibrated mRNA transcripts into 2  $\mu$ g of a host total RNA, 0.75  $\mu$ g of oligo (dT)<sub>18</sub> (0.27  $\mu$ g for *Fusarium* reactions), and 10 mM of dNTP mix. The volume was adjusted by water to 13  $\mu$ l, then mixed well and incubated at 65 °C for 5 min. The reaction tubes were chilled on ice at least for 1 min and the following was added: 4  $\mu$ l 5 $\times$  first strand buffer, 1  $\mu$ l 0.1 M DTT, 1  $\mu$ l SuperScript III (200 U/ $\mu$ l) (Invitrogen, CA), and 1  $\mu$ l RNaseOUT (40 U/ $\mu$ l) (Promega, WI). The final volume of the reaction was 20  $\mu$ l. The volume of this reverse transcription reaction can be proportionally enlarged to 80  $\mu$ l for consistent performance in our laboratory. The reaction was incubated at 50 °C for 1 h, 70 °C for 15 min, and 4 °C to end the reaction using a PCR cycler.

SYBR Green iTaq PCR master mix (BioRad Laboratories) was applied for each qRT-PCR reaction. For each reaction, a total of 25  $\mu$ l was used consisting of 12.5  $\mu$ l 2X SybrGreen MasterMix, 0.5  $\mu$ l each of forward and reverse primer (10  $\mu$ M each), 0.25  $\mu$ l cDNA template and 11.25  $\mu$ l H<sub>2</sub>O. On each 96-well plate, reactions of qRT-PCR were carried out with five replications for each control gene (four replications for a negative control *B2M*) and three replications for each target gene. For a second set of tests for the two species on the same ABI qRT-PCR 7500 system, three replications were carried out for each of the control genes and test target genes. On the ABI Sequence Detection 7500 System, PCR reactions were carried out with the following thermal profile: stage1: 95 °C for 3 min; stage 2: 40 cycles of 95 °C for 15 s and 60 °C for 45 sec; stage 3: 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s; stage 4: run dissociation curve with 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Stat Collection was set at stage 2 step 2 (60 °C for 45 s). A separated dis-

**Table 1**

Primers of universal RNA controls and selective target genes used for absolute mRNA quantification using SYBR Green by real time qRT-PCR.

ID	Sequence 5'–3'	Amplicon (bp)	Tm	Gene
CHA1.L CHA1.R	GTGCCAGGTTATCTGTGACT AATGGGCTCAATGACCTGAG	101	75.23	CHA1
Snq2.L Snq2.R	TATCAAAAGCTGGCCAATCC GTTTGTCCACCTTCTCTCAA	103	76.30	SNQ2
PDR3.L PDR3.R	ATACTGCCGAACGGAGAAGA CTGAAATCCTTCGGCAAGAG	130	73.30	PDR3
PDR5.L PDR5.R	GTACCGTGGTTGGAGCTGTT GAAACCACGCCATTGTCTT	101	76.80	PDR5
ADH7.L ADH7.R	ATTTCCAACGCAAAGGATTG AGATCCGCAGATACCACAGG	111	77.60	ADH7
Y63.L Y63.R	GGATTCTGTTTTGGTCCTCA ACGTCACGCACGTCAATAAA	147	76.80	Y63
GPM1.L GPM1.R	ATTGTCTGCCAAGGGTCAAC CTGTCAGCCTTTTCCAAGC	141	80.00	GPM1
RPN5.L RPN5.R	ATTCGCTCGCTCAAAATGAT CTGGCCAAAACCTCTTTCG	110	77.80	RPN5
TEF1a.L TEF1a.R	GTACGCCTGGGTTCTTGACA GAGCGTCTGGTAGGCATGTTA	165	82.53	TEF1a
β-tubulin.L β-tubulin.R	GCCATGAAGGAGGTTGAGGA AAGCCTTGCGTCGGAACATA	217	88.60	β-tubulin
TRI1.L TRI1.R	GATCACATAACCCACGCCATTG AATTCCGACGCCACTCTTGGT	179	83.07	TRI1
TRI4.L TRI4.R	GCCACTGCTGCTACTGTGTA GGTCGTTGTCCAGATGTTCTTG	157	82.93	TRI4
TRI5.L TRI5.R	TGGAGAACTGGATGGTCTGG GACATAGCCGTGCATGAAGC	239	84.23	TRI5
TRI13.L TRI13.R	CTGCGGTGGAACAGATGGTA ACACTGGCGTGTCCGTAAAG	178	83.27	TRI13
TRI15.L TRI15.R	ACTGCCACTGCCACAACAAG AGAGGCATTGGCCTGGTGTA	151	85.20	TRI15
TRI101.L TRI101.R	ATCGCCAACGAACCACTTG TGATGCTGCTTGACGGATTTC	162	84.97	TRI101

sociation curve assay was performed for each reaction to confirm gene-specific amplification.

Reactions in yeast RNA background were performed on an ABI 7500 system. For reactions in the Fusarium RNA background, an MJ PTC-200 DNA Engine with a Real Time Detector Chromo4 Opticon Monitor 3 was originally applied using version 3.1 software (Bio-Rad, Hercules, CA). However, since the PCR amplification efficiency was found to be lower on the Chromo4, the final confirmation of the Fusarium reactions were performed on the ABI 7500 system with above mentioned conditions. For the Chromo4 system, the following conditions were used: 95 °C for 10 min, 95 °C for 15 s, 60 °C for 45 s, plate read, 39 repeat cycles. The last cycle was 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. A melting curve ran from 55 °C to 95 °C, reading every 0.2 °C. All samples had only a single peak, indicating a pure product and no primer/dimer formation. Amplicons of a single band were also confirmed by agarose gel electrophoresis. On each instrument, a total of 12 96-well plate runs was done for each species.

## 2.6. Data analysis

At the end of each reaction run, data were exported to an Excel file and treated using a custom programmed macro visual basic function. Statistical analyses were done using the Excel statistical function tools. In addition, a general linear model experimental design weighted regression was applied for data analysis and confirmation. Full and reduced models were used to determine if there

were significant differences between the equations for each treatment condition for each plate at  $p \leq 0.008$  (dividing  $p$ -value of 0.5 by 6 F tests). Analyses were performed using TableCurve 2D Version 5.00 (TableCurve (2000)) and SAS PC Windows Version 9.1.3 software (Neter et al., 1990; SAS, 2003). PCR amplification efficiency for each reaction run was calculated as previously described (Applied Biosystem, 2006; Liu and Saint, 2002; Livak and Schmittgen, 2001). After data validation, we developed an automatic process for raw data handling using C++ program. The executable MasterqRT-PCR program performs comprehensive tasks of the control system as described in this study are available to qualified users by request. It is also accessible at <http://cs1.bradley.edu/~nri/MasterqRT-PCR>.

## 3. Results

### 3.1. CAB as a sole reference of manual threshold for data analysis

As anticipated, consistent performance of each replicated control genes was highly reproducible for the qRT-PCR. Each of the five replicated reactions almost overlaid and little variation was observed (Fig. 1). The performance of each control gene was independent from the toxic HMF treatment for *S. cerevisiae* or varied incubation time conditions for *F. sporotrichioides*. Mean value of the five CAB amplifications on a plate was designated as a constant reference to set-up a manual threshold at cycle number (Ct) 26 (Fig. 1). This sole reference served as a constant reference of data acquisition and analysis for every qRT-PCR run.

Highly fitted linear relationships were observed for the replicated qRT-PCR tests cross varied treatment conditions for *S. cerevisiae* and *F. sporotrichioides* (Table 2). In the *S. cerevisiae* RNA background, standard curves constructed under a control and HMF-treated conditions were similar and no significant variations were found when the reactions were performed on the ABI 7500 system (Fig. 2 and Table 2). Similar results were obtained for the Fusarium RNA background using ABI 7500 system, (Table 2). However, these reactions performed on the Chromo4 System showed lower amplification efficiency as measured by a slope (data not shown). Data generated on the Chromo4 were not used and only those obtained from the ABI 7500 system were used for analysis.

### 3.2. Master equations of standard curves

A standard curve was constructed for each treatment condition of a qRT-PCR run. A total of 36 individual standard curves were obtained for the yeast and 18 for the Fusarium reactions. Since there was no significant variation observed among individual standard curves for the yeast RNA and Fusarium RNA background under varied conditions, a master equation of standard curves was obtained using the total sum of data regardless of treatment conditions for the yeast and Fusarium reactions, respectively, as follows:

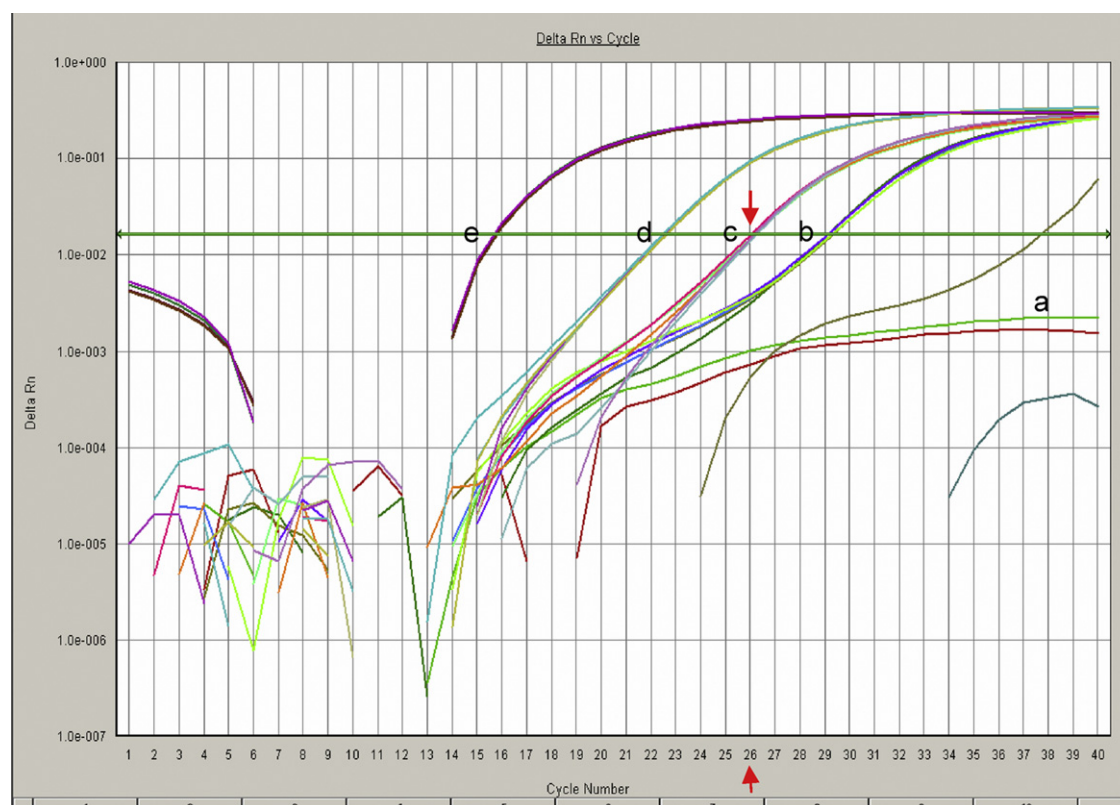
$$Y = 25.67 - 3.3508X \quad (R^2 = 0.9982) \quad (1)$$

$$Y = 25.55 - 3.4619X \quad (R^2 = 0.9959) \quad (2)$$

where  $X$  represents log mRNA (pg), and  $Y$  equals qRT-PCR cycle number (Ct) performed on the ABI qRT-PCR 7500 system. The master equation (1) was applied for *S. cerevisiae* reactions regardless of the control or HMF-treated conditions. The master equation (2) was for the *F. sporotrichioides* RNA background reactions regardless of the cultivation period of time.

### 3.3. PCR amplification efficiency

As measured by a slope of the linear correlation equations, PCR amplification efficiency for the yeast performed on the ABI



**Fig. 1.** A typical amplification plot of five control genes on an ABI qRT-PCR 7500 system showing performance of a four replicated non-template negative control *B2 M* (a), and five replicated each of *MSG*, *CAB*, *RBS1*, and *ACTB* at 0.1 (b), 1 (c), 10 (d), and 1000 pg (e), respectively. The sole reference of *CAB* (c) was designated to serve as a manual threshold at 26 Ct (indicated by arrows) for a constant data acquisition and analysis for each qRT-PCR run.

7500 system showed consistently high efficiency. For individual standard curves, values of the slope of the different test sets ranged from  $-3.38$  to  $-3.31$  reflecting amplification efficiency of 98–100% (Tables 2 and 3). When a master equation was used for *S. cerevisiae* with a slope of  $-3.35$ , the PCR amplification efficiency reflected an average amplification efficiency of 99% for the control. The amplification efficiency in *F. sporotrichioides* showed 95% when the master equation was applied with a slope of  $-3.46$  (Tables 2 and 3).

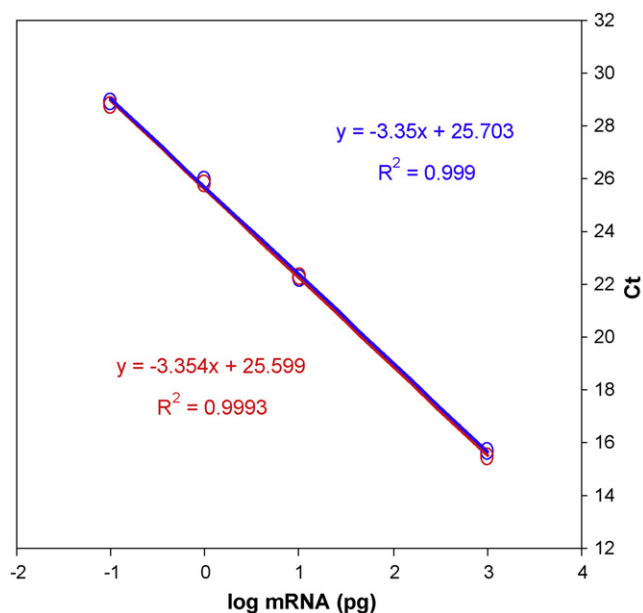
### 3.4. Data acquisition using options of auto vs. manual threshold

Comparisons were made using the options of the Auto threshold provided by the manufacture's built-in program and a manual set-up using *CAB* at a fixed value of Ct 26. Both the control genes and the selective target genes were used in multiple replicated plate reactions. The data obtained for all genes tested using the sole *CAB* reference setting at a fixed value of the Ct 26 showed significantly less variations compared with those obtained by the Auto option

**Table 2**

Consistent performance of the newly developed quality control system applying *CAB* as a manual threshold and a master equation in qRT-PCR assays under a control and toxic HMF treatments for *Saccharomyces cerevisiae* and varied cultural conditions (16 h vs. 24 h) for *Fusarium sporotrichioides* performed on an ABI real time PCR 7500 unit.

Test set	Slope				Intercept				R <sup>2</sup>	
	Control	Stdev.	HMF-treated	Stdev.	Control	Stdev.	HMF-treated	Stdev.	Control	HMF
<i>Saccharomyces cerevisiae</i>										
1	-3.3396	0.0075	-3.3602	0.0574	25.61	0.1817	25.56	0.2192	0.9968	0.9958
2	-3.3526	0.0017	-3.3554	0.0089	25.62	0.1407	25.55	0.3486	0.9976	0.9985
3	-3.3539	0.0174	-3.3618	0.0335	25.73	0.1994	25.67	0.3592	0.9982	0.9992
4	-3.3551	0.0301	-3.3817	0.0116	25.81	0.1541	25.76	0.5162	0.9991	0.9997
5	-3.3369	0.0015	-3.3556	0.0021	25.76	0.1612	25.70	0.4745	0.9986	0.9996
6	-3.3502	0.0269	-3.3086	0.0182	25.75	0.1541	25.58	0.4434	0.9988	0.9989
Mean	-3.3500	0.0142	-3.3539	0.0220	25.70	0.1652	25.60	0.3935	0.9982	0.9986
Master equation	-3.3508				25.67				0.9992	
<i>Fusarium sporotrichioides</i>										
Test	Condition 1 @ 16h	Stdev.	Condition 2 @ 24h	Stdev.	Condition 1 @ 16h	Stdev.	Condition 2 @ 24h	Stdev.	Condition 1 @ 16h	Condition 2 @ 24h
1	-3.4889		-3.4940		25.46		25.75		0.9995	0.9980
2	-3.4914		-3.4563		25.46		25.63		0.9967	0.9941
3	-3.4596		-3.3807		25.69		25.31		0.9948	0.9922
Mean	-3.4730	0.0177	-3.4437	0.0577	25.54	0.1334	25.56	0.2280	0.9970	0.9948
Master Equation	-3.4619				25.55				0.9959	

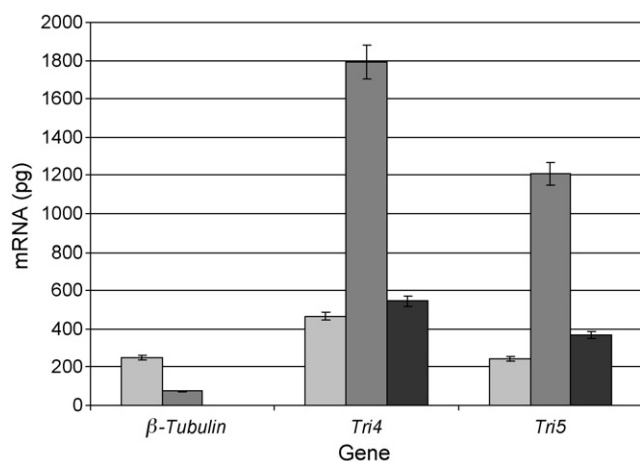


**Fig. 2.** Performance of the control genes as measured by standard curves under varied experimental conditions. Standard curves constructed using the four well calibrated control genes of *MSG*, *CAB*, *RBS1*, and *ACTB* in *Saccharomyces cerevisiae* RNA background under a control (blue) and toxic 5-hydroxymethylfurfural treatment (red) showing consistent performance of the control genes independent from the toxic challenges.

(Table 4). This low variation was consistent under the control or the HMF-treated toxic conditions for the yeast reactions. Similar results were obtained for the *Fusarium* RNA reactions (data not shown).

### 3.5. Normalization using the housekeeping gene vs. the robust mRNA reference

The conserved housekeeping gene  $\beta$ -tubulin showed reduced mRNA abundance at 24 h compared with that at 16 h. When it was used as an internal control to normalize the differentially expressed data at 24 h, gene *Tri4* and *Tri5* appeared only slightly higher in mRNA abundant compared with those observed at 16 h (Fig. 3). In contrast, their mRNA abundance was significantly higher at 24 h



**Fig. 3.** Comparison of mRNA estimates using housekeeping gene  $\beta$ -tubulin and the master equation for expression of selective genes of *Fusarium sporotrichioides* in response to different culture conditions. The estimated mRNA abundance using the master equation at 16 h and 24 h is shaded in light-gray and gray, respectively. The mRNA estimates at 24 h normalized by the housekeeping gene  $\beta$ -tubulin are dark-shaded.

**Table 3**

PCR amplification efficiency as indicated by a slope from linear regression relationships.

Slope	Exponent amplification	Amplification efficiency <sup>a</sup>
-3.85 <sup>b</sup>	1.81861	0.81861 <sup>b</sup>
-3.84	1.82145	0.82145
-3.83	1.82430	0.82430
-3.82	1.82717	0.82717
...	...	...
-3.60	1.8957	0.8957
-3.59	1.8991	0.8991
-3.58	1.9025	0.9025
-3.57	1.9060	0.9060
-3.56	1.9094	0.9094
-3.55	1.9129	0.9129
-3.54	1.9164	0.9164
-3.53	1.9199	0.9199
-3.52	1.9235	0.9235
-3.51	1.9271	0.9271
-3.50	1.9307	0.9307
-3.49	1.9343	0.9343
-3.48	1.9380	0.9380
-3.47	1.9417	0.9417
-3.46	1.9454	0.9454
-3.45	1.9492	0.9492
-3.44	1.9530	0.9530
-3.43	1.9568	0.9568
-3.42	1.9606	0.9606
-3.41	1.9645	0.9645
-3.40	1.9684	0.9684
-3.39	1.9724	0.9724
-3.38	1.9763	0.9763
-3.37	1.9803	0.9803
-3.36	1.9844	0.9844
-3.35	1.9884	0.9884
-3.34	1.9925	0.9925
<b>-3.33</b>	<b>1.9966</b>	<b>0.9966</b>
<b>-3.32</b>	<b>2.0008</b>	<b>1.0008</b>
-3.31	2.0050	1.0050
-3.30	2.0092	1.0092
-3.29	2.0135	1.0135
-3.28	2.0178	1.0178
-3.27	2.0221	1.0221
-3.26	2.0265	1.0265
-3.25	2.0309	1.0309
-3.24	2.0354	1.0354
-3.23	2.0398	1.0398
-3.22	2.0444	1.0444
-3.21	2.0489	1.0489
-3.20	2.0535	1.0535
-3.19	2.0582	1.0582
-3.18	2.0628	1.0628
-3.17	2.0676	1.0676
-3.16	2.0723	1.0723
-3.15	2.0771	1.0771
-3.14	2.0820	1.0820
-3.13	2.0868	1.0868
-3.12	2.0918	1.0918
-3.11	2.0967	1.0967
<b>-3.10</b>	<b>2.1017</b>	<b>1.1017</b>

<sup>a</sup> Amplification Efficiency (E) was calculated using equation  $E = [10^{(-1/\text{slope})}] - 1$ .

<sup>b</sup> PCR amplification efficiency for qRT-PCR with a 100% was indicated by bolded numbers, 95–99% in normal font, 90–95% in italic, and non valid in gray shading.

than at 16 h for these two genes when the robust RNA control and the master equation were applied for normalization.

## 4. Discussion

Using yeast and a *Fusarium* fungus as examples, we demonstrated robust performance of *CAB* as a sole reference under varied treatment conditions and thereafter the extended use of a master equation for absolute mRNA quantification assays. This is the first development of a robust standard to serve as a constant reference for a manual threshold setting such as 26 Ct using *CAB*, for qRT-

**Table 4**

Comparison of cycle number (Ct) variations applying the Auto option or the Manual threshold option using the CAB setting at 26 Ct for data acquisition in multiple runs of qRT-PCR in *Saccharomyces cerevisiae* RNA background.

Gene	Condition 1 (control)				Condition 2 (HMF)			
	Auto		Manual		Auto		Manual	
	Ct	stdev	Ct	stdev	Ct	stdev	Ct	stdev
Control gene								
MSG	30.33	1.197	28.98	0.264	30.40	1.217	28.87	0.356
CAB	27.29	1.218	26.06	0.502	27.19	1.142	25.89	0.358
RBS1	23.47	1.404	22.31	0.206	23.64	1.036	22.40	0.306
ACTB	16.34	1.090	15.69	0.184	16.08	1.064	15.51	0.270
Target gene								
CHA1	25.90	1.559	23.99	0.429	21.65	1.800	19.69	0.408
SNQ2	20.33	1.360	19.04	0.235	16.38	1.325	15.63	0.158
PDR3	22.83	1.592	21.38	0.419	17.59	1.120	16.69	0.186
PDR5	21.40	1.234	20.28	0.537	21.42	1.638	19.97	0.322
ADH7	23.97	1.757	22.49	0.540	18.45	1.735	17.08	0.242
Y63	20.51	1.291	19.34	0.386	18.72	1.354	17.61	0.324
GPM1	16.10	1.104	15.47	0.119	16.83	1.292	15.99	0.178
RPN5	19.82	1.488	18.59	0.226	18.79	1.638	17.47	0.292

PCR data acquisition and analysis. The concept and development of the master equation for qRT-PCR application is novel. The master equation can be used to estimate target genes for the entire qRT-PCR experiment set. Once the robust master equation is established, no repeated reactions of the control genes are needed to construct standard curves on every 96-well plate. Many reaction wells can be saved for target gene test. Such a development simplifies conventional procedures of qRT-PCR, safeguards the data comparability, increases data reliability, reproducibility, and throughput of the assay.

As demonstrated by the overall individual standard curves under different conditions for *S. cerevisiae* and *F. sporotrichioides* between different experimental data sets, linear fitness of the standard curve is reproducible and robust. When a well calibrated transcript of CAB is used to set-up a manual Ct threshold at 26 for qRT-PCR data acquisition and analysis, this master equation can be used as a representative standard reference to evaluate absolute mRNA mass. In conventional practice for qRT-PCR data acquisition, the threshold set-up is rather arbitrary by users. For example, there are two options available using the ABI 7500 System, the Auto and the Manual. Under the Auto option, a default value is taken based on overall reaction performance on a 96-well plate. Such a value varies each time upon composition and performance of the tested target genes on the plate. For the Manual option, users will have to set-up a threshold aiming the approximate midpoint of the linear phase of overall reactions. The Auto option is often used when a user has difficulty to choice between the two. In either case, a relative reference can be used to normalize all genes on the entire plate. However, it is difficult to repeat the same threshold for multiple runs and such obtained results are often difficult to reproduce and compare each other. This is particularly true when assays are performed under multiple conditions and multiple plate reaction runs.

As shown in this study, significantly higher levels of variations were observed by the Auto analysis option compared with the Manual option referenced by the CAB control. Therefore, we do not recommend using the Auto option. The sole reference CAB with an input of 1 pg mRNA among the other calibration standard, was applied to set-up a fixed threshold of 26 Ct as a manual threshold data analysis for each plate reaction run. Reactions with this pre-defined mRNA dose of CAB hit the middle of the linear phase at the defined threshold constantly. Thus, it can serve as a robust reference. Therefore, repeated data can be obtained using this robust reference over multiple reaction run and varied conditions. As reported in this study, variation of master equation for the reference

controls is within the expected range ( $p \leq 0.05$ ). Full and reduced linear models further confirmed the validity of a master equation with variations at  $p \leq 0.008$  (dividing  $p$ -value of 0.5 by 6 F tests) such as in the yeast RNA background application.

Housekeeping gene  $\beta$ -tubulin has been identified as an internal reference for normalization of gene expression measurement (Alexander et al., 2004). However, its mRNA abundance varied at 24 h compared with that at 16 h as shown in this study. Normalized by  $\beta$ -tubulin, target genes *Tri4* and *Tri5* merely showed insignificant or marginal difference for their expression levels comparing 24 h and 16 h cultivation. Such derived interpretation would be misleading since these genes are known to be highly expressed at 24 h (Alexander et al., 2004). As measured by the master equation, the mRNA abundance for *Tri4* and *Tri5* showed anticipated increased expression of more than 4–5 times at 24 h compared with 16 h.

Comparison of mRNA expression is commonly presented by relative fold changes in the community. Using the robust mRNA control and the master equation in this study, expression of a gene was estimated in mRNA mass abundance. These values can be used directly without further normalizations in fold-change comparisons. For quantitative gene expression, it can simply use the mRNA mass in pg as shown by this study. Alternatively, the mRNA mass can be further converted easily to a gene copy number using an equation adapted from Staroscik (2004) as follows:

$$\text{Gene copy number} = \frac{[\text{mRNA}(\text{pg}) \times 6.022 \times 10^{20}]}{[\text{Amplicon}(\text{bp}) \times 1 \times 10^9 \times 650]} \quad (3)$$

where mRNA is an estimated value in pg using the master equation and Amplicon is the amplified bp-length of an interested target gene. The capability to access absolute mRNA abundance for each gene is an obvious advantage using the master equation when this option is desired.

PCR amplification efficiency has been shown as an increased concern for qRT-PCR performance recently. Numerous algorithms focused on PCR amplification efficiency have been developed (Liu and Saint, 2002; Livak and Schmittgen, 2001). However, since PCR amplification efficiencies vary significantly among genes within the same genome, calculation of amplification efficiency for each gene does not resolve assay performance problems. Nonetheless, adequate amplification efficiency for control genes is necessary to normalize overall reactions in a RNA background on a 96-well plate. PCR amplification efficiency for the control genes in this study was high (99%) as measured by the slope of the master equation on the ABI 7500 system in the yeast RNA background reactions. This indicated an independent performance of the control genes from the different toxin treatment. Based on the master equation, the amplification efficiency in *F. sporotrichioides* RNA background performed on the ABI 7500 system was 95% as reflected by a slope of  $-3.46$ . Amplification efficiency of standard gene expression assay using qRT-PCR was anticipated at a variation range of  $100 \pm 10\%$  (Applied Biosystem, 2006). As measured by the Ct slope method, such values fall between  $-3.58$  and  $-3.11$ . Independent experimental data of the control from this study were observed to have amplification efficiencies of 99% and/or 95% for yeast and *Fusarium*, respectively. Such amplification efficiencies are within the variation ranges of the qRT-PCR assay. We conclude that the robust CAB manual threshold setting and the master equation developed in this study on the ABI 7500 system are valid reference for the qRT-PCR assay.

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